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Patents ADP number *(if you know it)*
6254007002

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This invention relates to antisense oligodeoxynucleotides targeted to sequences in thymidylate synthase (TS) mRNA. In particular the invention relates to antisense oligodeoxynucleotides targeted to sequences in the 3' end of TS mRNA, which antisense oligodeoxynucleotides are both cytostatic on their own when administered to human tumour cell lines, and also enhance the toxicity of the anticancer drugs such as Tomudex administered to those cells. In contrast, antisense oligodeoxynucleotides targeted to sequences at or near the translation start site at the 5' end of TS mRNA have either no effect, or enhance cell growth, when administered on their own. In addition, antisense nucleic acids targeted to these 5' sequences (but not to 3' sequences) induce TS gene transcription. The invention also relates to a combination product comprising an antisense oligodeoxynucleotide in combination with an anticancer agent such as Tomudex (N-(5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thenoyl)-L-glutamic acid) or the Zeneca development compound ZD 9331 ((S)-2-(2-fluoro-4-[N-(4-hydroxy-2,7-dimethylquinazolin-6-ylmethyl)-N-(prop-2-ynyl)amino]benzamido)-4-(1H-1,2,3,4-tetrazol-5-yl)butyric acid), and to the use of such a combination product in the treatment of cancer.

Thymidylate synthase (TS) (EC 2.1.1.45) catalyses the conversion of deoxyuridylate ~~to thymidylate, and is a housekeeping enzyme essential for the only intracellular *de novo*~~ synthesis of thymidylate (Danenberg, 1977). TS gene expression is tightly regulated with respect to cell proliferation state (Maley and Maley, 1960; Lochsin *et al.*, 1979). As such, the TS gene is part of a group of genes whose expression is elevated at the G_1/S cell cycle boundary, and it has been suggested that transcription of several S-phase genes (including dihydrofolate reductase and thymidine kinase) is controlled in part by the E2F family of transcription factors (Farnham *et al.*, 1993; Mudrak *et al.*, 1994). In fact, transfection of active E2F1 genes into mouse cells induces expression of TS and other S-phase and cell cycle-regulated genes (De Gregori *et al.*, 1995). As cells progress through the cell cycle from G_0 through S phase, TS mRNA levels increase approximately 20-fold and TS enzyme activity increases about 10-fold (Navalgund *et al.*, 1980). However, TS gene transcription rate is upregulated only 2 to 4 times, suggesting that post-transcriptional events play a major role in TS regulation (Ayusawa *et al.*, 1986; Jenh *et al.*, 1985; Johnson, 1994). Differences in TS

mRNA stability are not likely to be critical in regulation, as TS mRNA half-life is about 8 hours in both resting and growing rodent cells (Jenh *et al.*, 1985). On the other hand, TS mRNA translation appears to be regulated by the TS protein itself, which specifically interacts at two sites within its own mRNA to inhibit protein production (Chu *et al.*, 1991, 1993b, 1994; Voeller *et al.*, 1995). Translation of other mRNAs (including c-*myc* mRNA) may also be regulated by interactions with TS protein (Chu *et al.*, 1995).

Because of its role in DNA precursor synthesis, TS has been identified as a potential target for cancer chemotherapeutic agents (Hardy *et al.*, 1987). High TS levels have been correlated with poor prognosis in patients with ovarian cancer (Suzuki *et al.*, 1994), rectal cancer (Johnston *et al.*, 1994) childhood acute non-lymphoblastic leukaemia (Volm *et al.*, 1994), and non-small cell lung carcinoma (Volm and Mattern, 1992). However, its prognostic value is not high in all tumour types (Peters *et al.*, 1986, 1994). Two types of TS inhibitors have been developed: (a) nucleotide analogues (including 5-FU, its riboside, and deoxyriboside derivatives) which must be activated to 5-fluorodeoxyuridylate (FdUMP) within cells to be effective (Heidelberger *et al.*, 1983) and (b) 5,10-CH_2FH_4 (antifolate) analogues, including *N*-10-propargyl-5,8-dideazafolate (CB3717) (Calvert *et al.*, 1986) and Tomudex (ZD1694; *N*-[5-(*N*-[3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl]-*N*-methylamino)-2-thenoyl]-L-glutamic acid) (Jackman *et al.*, 1991a, 1991b). Although Tomudex and 5-FU inhibit TS and have potent cytotoxic and antitumour activity (Heidelberger *et al.*, 1983; Keyomarsi *et al.*, 1993), they have an unusual biochemical effect. When human cancer cell lines are treated with 5-FU or Tomudex, TS levels increase rapidly, perhaps as a result of the release of translational inhibition by the TS protein (Keyomarsi *et al.*, 1993; Chu *et al.*, 1990; Chu *et al.*, 1993a).

It has been speculated that the release of translational inhibition that accompanies binding and inactivation of TS by chemotherapeutic agents (including Tomudex and 5-FU) might be prevented by treating cells with agents that could replace the specific interaction between TS mRNA and TS protein, and inhibit translation (Keyomarsi *et al.*, 1993) but no such agents were described. In another speculative article it was hypothesised that antisense nucleic acids designed to both reduce the ability of TS mRNA to direct protein production, and to interact with the TS protein binding site, may be useful in complementing the effectiveness of drugs targeted against TS (Rapaport *et al.*, 1992).

In previous patent applications, UK 9720107.3 and UK 9722012.3, we disclosed how to specifically down-regulate the expression of TS in human breast cancer (MCF-7) cells in two ways. First, we both transiently and stably transfected the cells with vectors expressing antisense RNA molecules directed to hybridise to three different regions of the TS mRNA. Targeted sequences were: (1) sequences participating in the formation of a putative stem-loop structure surrounding the translation start site, and immediately adjacent and 3' to that site (these sequences also participate in binding TS protein to modulate translation), (2) the exon1/exon2 boundary and (3) the 3' end of the mature cytoplasmic mRNA. Antisense TS RNA was expressed from these vectors (as assessed by northern blot analysis and a novel modification of the run-on transcription assay to measure antisense transcription against background constitutive TS gene expression) (Koropatnick *et al.*, 1997). Second, we transiently transfected cells with single-stranded oligodeoxynucleotides targeted to hybridise to: (a) the translation start site and sequences surrounding it, (b) a sequence proximal to the translation start site and participating in the putative stem-loop structure, and (c) the translation stop site near the 3' end of the mature cytoplasmic RNA.

The present invention is based on our discovery that an antisense oligonucleotide, oligo 83, complementary to a sequence in the TS mRNA 3' untranslated region, down-regulated the level of TS mRNA and protein, inhibited cell proliferation and enhanced the cytotoxicity of TS-directed chemotherapy drugs.

In a first aspect of the invention we provide an antisense oligodeoxynucleotide which hybridises to a target nucleic acid sequence in thymidylate synthase and which selectively inhibits thymidylate synthase production in mammalian cells. Preferably the oligonucleotide is targeted to sequences at or near the translational stop site at the 3' end of the TS gene, which sequences lie in the region between bases 800 and 1600, using the sequence numbering described for human thymidylate synthase mRNA by Takeishi *et al.*, 1985. More preferably the sequences lie in the region between bases 1000 and 1530. Most preferably the sequences lie in the region between bases 1030 and 1460.

In a second aspect of the invention we provide an antisense oligodeoxynucleotide which hybridises to a target nucleic acid sequence in thymidylate synthase and which selectively enhances thymidylate synthase production in mammalian cells. Preferably the oligonucleotide is targeted to sequences at or near the translation start site at the 5' end of the

TS gene, which sequences lie in the region between bases 1 and 300, using the sequence numbering in Takeishi *et al.*, 1985. More preferably the sequences lie in the region between bases 50 and 200. Most preferably the sequencs lie in the region between bases 90 and 130.

An antisense oligodeoxynucleotide is an oligonucleotide which is designed to hybridise to a specific region of a target nucleic acid sequence. The target nucleic acid is the TS gene or mRNA transcribed from the TS gene. Preferably the target nucleic acid is the mRNA encoding thymidylate synthase.

The effects of antisense oligonucleotides on thymidylate synthase expression can be measured using procedures which are well known to persons skilled in the art. In the present application, effects on mRNA levels have been measured by Northern blot analysis and nuclear run-on transcription assay, and effects on the growth of human tumour cells have been measured by counting cell numbers using a Coulter counter.

Antisense oligonucleotides to thymidylate synthase may inhibit, stimulate or have no effect on thymidylate synthase expression. Of these, preferred antisense oligonucleotides are those which either inhibit or stimulate thymidylate synthase expression, and particularly preferred antisense oligonucleotides are those which inhibit thymidylate synthase expression.

By inhibition of thymidylate synthase expression we mean inhibition of at least 10% relative to the untreated control, measured at day 4 using the assay described in Example 1.2. Preferably inhibition of thymidylate synthase expression is at least 20% and most preferably inhibition is at least 40%.

By stimulation of thymidylate synthase expression we mean stimulation of at least 10% relative to the untreated control, when measured at day 7 using the assay described in Example 1.2. Preferably stimulation is at least 20% and most preferably stimulation is at least 40%.

Preferably, the antisense oligonucleotides are from about 8 to about 50 nucleotides in length, more preferably from about 12 to about 40 nucleotides in length and most preferably from about 16 to about 30 nucleotides in length.

Specific examples of sequences of antisense oligonucleotides which regulate thymidylate synthase activity are shown in Table 1. The regions of TS mRNA targeted by the oligonucleotides are shown in Figure 7.

Table 1:

ANTISENSE OLIGONUCLEOTIDE	SEQUENCE
ODN 83	TTAAGGATGTTGCCACTGGC
ODN 32	ATGCGCCAACGGTTCCTAAA

It will be appreciated that the invention is not restricted merely to those specific antisense oligonucleotides which are disclosed in Table 1 above but encompasses oligonucleotides of from about 8 to about 50 nucleotides in length which selectively inhibit or selectively enhance thymidylate synthase production and which are selected from those regions of the TS gene which are described hereinbefore.

Hybridisation of an antisense oligonucleotide to its target nucleic acid sequence is mediated by the formation of hydrogen bonds between complementary bases on each nucleic acid strand. Hybridisation may occur between nucleic acid strands which have varying degrees of complementarity, depending on the hybridisation conditions employed. The term "specifically hybridisable" is used to describe an oligonucleotide which has a sufficient degree of complementarity to ensure stable, specific binding to its target sequence, whilst avoiding non-specific binding to non-target sequences.

Antisense oligonucleotides may be designed to hybridise to any region within the thymidylate synthase mRNA molecule, including the coding region, the 5'untranslated region, the 3'untranslated region, the 5'cap region, introns and intron/exon splice junctions.

Hybridisation of the antisense oligonucleotide to thymidylate synthase mRNA may affect any aspect of mRNA function, for example mRNA translocation, mRNA splicing, mRNA translation, or the feedback inhibition mechanism regulated by the binding of thymidylate synthase protein to binding sites within the thymidylate synthase mRNA molecule.

An oligonucleotide is a polymeric molecule which is assembled from nucleotide or nucleoside monomers. The monomers may consist of naturally occurring bases, sugars and inter-sugar linkages or may also contain non-naturally occurring derivatives which modify the

properties of the oligonucleotide, for example, phosphorothiorated oligonucleotides have been used in the present application to increase resistance to nuclease degradation.

Preferred oligonucleotides may contain phosphorothiorates, phosphotriesters, methyl phosphonates or short chain alkyl, cycloalkyl or heteroatomic intersugar linkages. Other preferred oligonucleotides may be methoxy-ethoxy winged or may contain a peptide nucleic acid backbone. Particularly preferred oligonucleotides are those containing phosphorothiorates (Summerton, J.E. and Weller, D.D., U.S. Patent No: 5,034,506).

The oligonucleotides may be manufactured using any convenient method of synthesis. Examples of such methods may be found in standard textbooks, for example "Protocols for Oligonucleotides and Analogues; Synthesis and Properties," Methods in Molecular Biology Series; Volume 20; Ed. Sudhir Agrawal, Humana ISBN: 0-89603-247-7; 1993; 1st Edition.

In a further aspect of the invention, there is provided a pharmaceutical composition comprising an antisense oligonucleotide targeted to thymidylate synthase as defined hereinbefore in a pharmaceutically acceptable diluent or carrier.

In a further aspect of the invention there is provided a method for the treatment of cancer (or a method for providing an antiproliferative effect) which comprises administering to a warm-blooded animal an effective amount of an oligonucleotide targeted to thymidylate synthase as defined hereinbefore. The invention also provides the use of such an oligonucleotide in the production of a new medicament for the treatment of cancer (or for the treatment of proliferative disease.

In a further aspect of the present invention, there is provided a combination product comprising an antisense oligonucleotide targeted to thymidylate synthase in combination with an anticancer agent. The antisense oligonucleotide and the anticancer agent may be administered separately, sequentially, simultaneously or in a mixture.

The anticancer agent may cover three main categories of therapeutic agent:

(i) thymidylate synthase inhibitors such as Tomudex (N-(5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thenoyl)-L-glutamic acid) (European Patent Application no. 0239362, Example 7, compound no. 8 therein); Zeneca development compound ZD9331 ((S)-2-(2-fluoro-4-[N-(4-hydroxy-2,7-dimethylquinazolin-6-ylmethyl)-N-

(prop-2-ynyl)amino]benzamido)-4-(1H-1,2,3,4-tetrazol-5-yl)butyric acid) (European Patent Application no. 0562734, Example 3 thereof); LY 231514 (Eli Lilly Research Labs, Indianapolis, IN); 1843U89 (Glaxo-Wellcome, Research Triangle Park, NC); AG337 and AG331 (both by Agouron, La Jolla, CA) (Touroutoglou and Pazdur, Clin. Cancer Res., 2, 227-243, 1996).

(ii) cytostatic agents such as antioestrogens (for example tamoxifen, toremifene, raloxifene, droloxifene, iodoxyfene), progestogens (for example megestrol acetate), aromatase inhibitors (for example anastrozole, letrazole, vorazole, exemestane), antiprogestogens, antiandrogens (for example flutamide, nilutamide, bicalutamide, cyproterone acetate), LHRH agonists and antagonists (for example goserelin acetate, luprolide), inhibitors of testosterone 5α-dihydroreductase (for example finasteride), anti-invasion agents (for example metalloproteinase inhibitors like marimastat and inhibitors of urokinase plasminogen activator receptor function) and inhibitors of growth factor function, (such growth factors include for example EGF, FGFs, platelet derived growth factor and hepatocyte growth factor such inhibitors include growth factor antibodies, growth factor receptor antibodies, tyrosine kinase inhibitors and serine/threonine kinase inhibitors).

(iii) antiproliferative/antineoplastic drugs and combinations thereof, as used in medical oncology, such as antimetabolites (for example antifolates like methotrexate, fluoropyrimidines like 5-fluorouracil, FUdR, ftorafur, FdUR, purine and adenosine analogues, cytosine arabinoside); antitumour antibiotics (for example anthracyclines like doxorubicin, daunomycin, epirubicin and idarubicin;) mitomycin-C, dactinomycin, mithramycin; platinum derivatives (for example cisplatin, carboplatin, oxaliplatin); alkylating agents (for example nitrogen mustard, melphalan, chlorambucil, busulphan, cyclophosphamide, ifosfamide, nitrosoureas, thiotepa); antimitotic agents (for example vinca alkaloids like vincrisitine and taxoids like taxol, taxotere); topoisomerase inhibitors (for example epipodophyllotoxins like etoposide and teniposide, amsacrine, topotecan).

The anticancer treatment may also be radiotherapy.

Particularly preferred anticancer agents are thymidylate synthase inhibitors such as Tomudex (N-(5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thenoyl)-L-glutamic acid) (European Patent Application no. 0239362, Example 7, compound no. 8 therein) and the Zeneca development compound ZD9331 ((S)-2-(2-fluoro-4-[N-(4-hydroxy-2,7-dimethylquinazolin-6-ylmethyl)-N-(prop-2-ynyl)amino]benzamido)-4-(1H-1,2,3,4-tetrazol-5-yl)butyric acid) (European Patent Application no. 0562734, Example 3 thereof).

In a further aspect of the present invention there is provided a pharmaceutical composition comprising a combination product as defined hereinbefore and a pharmaceutically acceptable diluent or carrier.

Any pharmaceutical composition as defined hereinbefore may be in a form suitable for oral use, for example a tablet, capsule, aqueous or oily solution, suspension or emulsion; for topical use, for example a cream, ointment, gel or aqueous or oily solution or suspension; for nasal use, for example a snuff, nasal spray or nasal drops; for vaginal or rectal use, for example a suppository; for administration by inhalation, for example as a finely divided powder such as a dry powder, a microcrystalline form or a liquid aerosol; for sub-lingual or buccal use, for example a tablet or capsule; or particularly for parenteral use (including intravenous, subcutaneous, intramuscular, intravascular or infusion), for example a sterile aqueous or oily solution or suspension. In general the above compositions may be prepared in a conventional manner using conventional excipients.

Tomudex is conveniently administered to humans by intravenous injection of a sterile aqueous solution at a dose in the range, for example, of 1 to 4 mg/m^2 of body surface area once every three weeks, preferably at a dose of 3 mg/m^2 once every three weeks.

ZD 9331 is conveniently dosed to humans by oral administration of a solid dosage form or by intravenous injection of a sterile aqueous solution. The oral dosage form is conveniently administered to humans at a total dose in the range, for example, of about 1 to 100 mg/kg (i.e. about 35 mg/m^2 to 3.5 g/m^2) every three weeks given by a continuous or an intermittent dosing schedule, for example a dosing schedule of a three week dosing cycle comprising daily doses on days 1 to 5 only followed by no further doses until the next dosing cycle or a dosing schedule of a four week dosing cycle comprising daily doses on days 1 to 14 only followed by no further doses until the next dosing cycle. Preferably the oral dosage form

is administered to humans at a total dose in the range, for example, of about 1 to 30 mg/kg every three or four week dosing cycle. The sterile aqueous solution is conveniently administered intravenously to humans at a total dose of up to 100 mg/m^2 every three weeks given by a continuous or an intermittent dosing schedule, for example, a dosing schedule of one dose per three week dosing cycle, a dosing schedule of a three week dosing cycle comprising daily doses on days 1 to 5 only followed by no further doses until the next dosing cycle, a dosing schedule of a three week dosing cycle comprising doses on days 1 and 8 only followed by no further doses until the next dosing cycle or a dosing schedule of a three week dosing cycle comprising continuous infusion on days 1 to 5 followed by no further dosing until the next dosing cycle. Preferably the sterile aqueous solution is administered intravenously to humans at a total dose in the range, for example, of about 20 to 50 mg/m^2 every three weeks given by a continuous or an intermittent dosing schedule as illustrated hereinbefore.

The antisense oligonucleotide is conveniently administered to humans by intravenous injection of a sterile aqueous solution at a dose per dosing cycle in the range, for example, of 0.1μg to 1g, preferably at a dose of 1mg to 100mg.

The amount of active ingredient that is combined with one or more excipients to produce appropriate dosage forms will necessarily vary depending upon the particular component of the combination product, the host treated and the particular route of administration. For example, a formulation intended for oral administration to humans will generally contain, for example, from 0.5μg to 2g of active agent compounded with appropriate and convenient amounts of excipients which may vary from about 5 to about 98 percent by weight of the total composition. A formulation intended for parenteral administration to humans will generally contain 0.1μg to 50mg. Dosage unit forms will generally contain about 1μg to about 500mg of an active ingredient.

In a further aspect of the invention there is provided a method for the treatment of cancer (or a method for providing an antiproliferative effect) which comprises administering to a warm-blooded animal an effective amount of a combination product as defined above. The invention also provides the use of such a combination product in the production of a new medicament for the treatment of cancer (or for the treatment of proliferative disease).

Abbreviations used in this application are set out below.

TS	thymidylate synthase
CMV	cytomegalovirus
5-FU	5-fluorouracil
LFA	lipofectamine
PBS	phosphate-buffered saline
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
FBS	fetal bovine serum
MT	metallothionein
ODN	oligodeoxynucleotide
bp	base pairs
DMEM	Dulbecco's modified Eagle medium
oligo	oligonucleotide

The invention will now be illustrated but not limited by reference to the following Example and Figures wherein:

Figure 1: shows that antisense TS ODN 83 inhibits HeLa cell proliferation. HeLa cells transfected with 50 nM antisense TS ODN 83 (•) or 50 nM scrambled control ODN 32 (o) were assessed for cell proliferation at 1, 2, 5, and 6 days following transfection. Data points indicate the average of two measurements, and are representative of qualitatively similar results obtained in 16 independent experiments.

Figure 2 shows that antisense TS ODN 83 suppresses HeLa cell growth after transfection, followed by recovery to control proliferation rate after 48 hours. HeLa cells were transfected with 50 nM antisense TS ODN 83 or 50 nM scrambled control ODN 32 as described in the legend to Figure 1. Values derived from cells transfected with ODN 32 were normalised to 100%, and each bar indicates the percent of that value measured following treatment with ODN 83 (mean +- SE of 4 independent experiments). Asterisks (*) indicate significant differences ($p<0.02$, Student *t*-test).

Figures 3a & 3b show that treatment of HeLa cells with ODN 83 leads to decreased TS mRNA levels.

(Figure 3a) HeLa cells were transfected with ODN 83 or scrambled control ODN 32, or treated with Lipofectamine alone. Cells were harvested at 1, 2, and 4 days post-transfection and total cellular RNA isolated, reverse-transcribed, and TS and GAPDH cDNA amplified by 24 PCR cycles in the same reaction vessel. TS (208 bp) and GAPDH (752 bp) RT/PCR products were confirmed by Southern blotting and hybridisation to specific radioactively-labeled probes.

(Figure 3b) TS:GAPDH ratio of RT/PCR products from RNA isolated from HeLa cells 1 day after transfection with ODN 83 or ODN 32. Twenty-four, 25, 26, or 27 cycles of PCR amplification were carried out, revealing the same reduction in TS:GAPDH ratio after transfection with ODN 83.

Figure 4 shows that TS protein levels (inferred by measurements of 5-FdUMP binding) are diminished by antisense TS ODN 83 but not scrambled control ODN 32. 5-FdUMP binding was measured in cells transfected with ODN 83 (hatched bars) or ODN 32 (open bars) at different times following transfection.

~~(A): Results are plotted as a percent of 5-FdUMP binding in cells transfected with control~~ ODN 32+- SE *(n=5)*. The values for ODN 32 (n=5) were normalised to 100% and are shown without error bars.

(B): Results are presented as pmol 5-FdUMP bound per mg total protein (x 10^{-3}) to reveal that transfection with control ODN 32 had no significant effect on TS protein levels. Error bars indicate errors calculated according to a Student *t*-test, and indicate error due to differences in experimental conditions in 5 measurements taken on different days, and differences due to transfection with different ODNs. The asterisks indicate significant differences ($p<0.02$) determined by using a paired Student *t*-test.

Figure 5: shows that antisense TS ODN 83 sensitises HeLa cells to the toxic effects of 5-FU, 5-FUdR, Tomudex, and MTX, but not cisplatin or chlorambucil. HeLa cells were transfected with ODN 83 (•) or control ODN 32 (o) and treated with different concentrations

of 5-FU (A), 5-FUdR (B), Tomudex (C), MTX (D), cisplatin (E) or chlorambucil (F) for 4 days, beginning 24 h after transfection. Data points are plotted as the mean +- SE of 4 measurements. Where error bars are not apparent, they are obscured by the symbol. Asterisks (*) indicate significant differences <0.02, Student *t*-test).

Example 1

Example 1.1: Experimental Methods

Oligonucleotides:

Fully phosphorothioated 20-base oligonucleotides were synthesised by ISIS Pharmaceuticals (Carlsbad, California, USA). The 6 nucleotides on either end of the oligomer were methoxyethoxylated in the 2'-position, enhancing hybridisation as well as resistance to exonucleation. The middle 8 nucleotides were not methoxyethoxylated to allow RNase H endonucleation and degradation of mRNA hybridised to the oligomer. ODN 83 is complementary to TS mRNA, starting from a position 136 bases downstream of the translational stop site (5'-GCCAGTGGCAACATCCTTAA-3'). ODN 32 is a randomised sequence of ODN 83 (5'-ATGCGCCAACGGTTCCTAAA-3'), with the same base constituents in random order. A search of available mRNA sequences using the NCBI BLAST search tool revealed that ODN 83 had sequences of 10 or more complementary bases to only human TS mRNA, while ODN 32 had sequences of 10 or more complementary bases to no known mRNAs.

Radioisotope:

[6-^{3}H]5-FUdR (specific activity 18.6 Ci/mmol) was purchased from Moravek Biochemicals (Brea, California, USA). This isotope was 99.98% pure upon initial production, with a degradation rate of 0.5-1% per month at -20^{0}C, and was used within 3 months of manufacture.

Other supplies:

Cell culture chemicals and nutrients were obtained from Canadian Life Technologies (GIBCO) (Burlington, Ontario, Canada). All other chemicals were obtained from commercial sources. Plasticware was purchased from VWR Canlab (Mississauga, Ontario, Canada) and

Fisher Scientific Uniondale, Ontario, Canada).

Cell Culture:

Human cervical carcinoma HeLa cells were maintained in D-MEM plus 10% foetal bovine serum and penicillin (50 units/ml)/streptomycin (50 μg/ml). Cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C. Rapidly proliferating cells were utilised for establishing cultures of experimental cells, which were allowed to plate overnight prior to manipulation.

Transfection was performed using lipofectamine (LFA, GIBCO-BRL), a polycationic liposome formulation. Cells to be used for proliferation experiments were plated at a starting cell number of between 0.6 and 1 x 10^5 cells per 25-cm tissue culture flask, and LFA was used at 3 μg/ml. For cells in 75-cm flasks, which were to be harvested and extracted for assay of mRNA or TS content, the starting cell number was approximately 8 - 10 x 10^5, and the LFA concentration was 4 μg/ml. Prior to transfection, adherent HeLa cells were washed once with PBS and then treated with antisense or scrambled control ODN (50 nM) in the appropriate concentration of LFA in serum-free D-MEM, at 37°C for 6.0 h. The cells were then washed once with PBS and cultured in the presence of D-MEM plus 10% FBS. In cells treated with cytotoxic agents, exposure was initiated 24 hours after the removal of LFA/ODN, by addition of 0.2-volume of growth medium containing the agent at 6 times the final concentration. At the time of addition of drug, and after 4 days of incubation, cell numbers were determined from replicate flasks by enumerating with a particle counter (Coulter Electronics, Hialeah, Florida, USA). The proliferation of drug treated cells (fold-increase in cell number) was calculated as a percentage of that of the control cells. IC_{50} and IC_{90} values were determined by interpolation of plotted data.

RT-PCR to measure TS mRNA:

RNA was isolated from transfected cells using Trizol (GIBCO-BRL). Complementary DNA was synthesised from 1 μg of total RNA using 200 U of Moloney Murine Leukemia Virus reverse transcriptase (GIBCO BRL) in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1 mM mixed dNTPs, 100 pmol random primers and 10 mM dithiothreitol at 37°C for 1 hr. The enzyme was inactivated at 95°C for 5 min. The resulting cDNAs (in a volume of 2.5

μl) were amplified in a polymerase chain reaction (PCR) using 1.25 U of *Taq* DNA polymerase in 50 μl of 20 mM Tris-HCl pH 8.4), 50 mM KCl, 0.2 mM mixed dNTPs, 2 mM $MgCl_2$, and 50 pmol of primers specific for TS and GAPDH cDNAs. TS and GAPDH cDNAs were amplified together in the same reaction tube to allow the level of housekeeping GAPDH cDNA to be used to determine the relative level of TS mRNA. Twenty-four to 27 cycles of PCR amplification (94°C 45 s, 55°C 30 s, 72°C 90 s) produced fragments of 208 bp and 752 bp using primer sets for TS (forward 5'CACACTTTGGGAGATGCACA3'; reverse 5'CTTTGAAAGCACCCTAAACAOCCAT3') and GAPDH (forward 5'TATTGGGCGCCTGGTCACCA3'; reverse 5'CCACCTTCTTGATGTCATCA3'), respectively. PCR products were separated on a 1.2% agarose gel, and transferred to Hybond nylon membrane (Amersham, Canada, Ltd., Oakville, Ontario, Canada) by Southern blotting. Blots were hybridised to [α-^{32}P]dCTP random primer-labeled probe (pcHTS-1, a generous gift from Dr. K. Takeishi, University of Shizuoka, Shizuoka, Japan; or a cDNA insert recognising glyceraldehyde-3-phosphate dehydrogenase [GAPDH]). Hybridisation signals were quantified using a Phosphorlmager and ImageQuant (Molecular Dynamics, Sunnyvale, California, USA).

TS binding assay:

Cellular content of TS was assayed by binding of [6-^{3}H]5-FdUMP. This method was demonstrated to label total TS unless the cells were pretreated with 5-FU or 5-FUdR. The assay was performed using cells that were treated with antisense ODN 83 or the scrambled control ODN 82. Briefly, cells were harvested by scraping into PBS and resuspending the subsequent pellet in 100 mM KH_2PO_4 (pH 7.4). Cells were disrupted by freezing and thawing, followed by sonication. The total protein concentration was determined using Coomassie staining (BioRad reagent) (MI) in order to express results as pmol 5-FdUMP bound per mg total protein. 5-FdUMP binding was assessed in paired lysates from cells transfected with ODN 83 or ODN 32, in separate incubation reactions carried out on different days; however, pairs were always assessed together under the same reaction conditions. On each occasion, the incubation vessel contained 50 μg of total protein, 75 μM methylene-FH_4; 100 mM mercaptoethanol, 50 mM KH_2P0_4 (pH 7.4), and 15 nM [6-^{3}H]5-FdUMP in a final volume of 200 μl. After 30 min at 37°C, the incubation was stopped by addition of 5 volumes

of albumin-coated, activated charcoal. After 10 min (room temperature), this slurry was centrifuged (3000 x g, 30 min, 22^{0}C), and the supernatant re-centrifuged to completely remove particulate matter. Two aliquots of 300 μl each were removed from the final, clarified supernatant for scintillation counting.

Statistical analysis:

Data for cell growth after treatment with ODNs alone, or in combination with cytotoxic drugs, are presented as the mean +/- standard error or standard deviation as determined by Student *t*-test. For determinations of FdUMP binding, differences between paired samples from cells transfected with different ODNs were assessed using a paired *t*-test. This controlled for differences in experimental conditions on each of the 5 occasions that FdUMP binding was assessed. In all cases, significance was chosen *a priori* to be indicated by differences at a confidence level of $p<0.02$.

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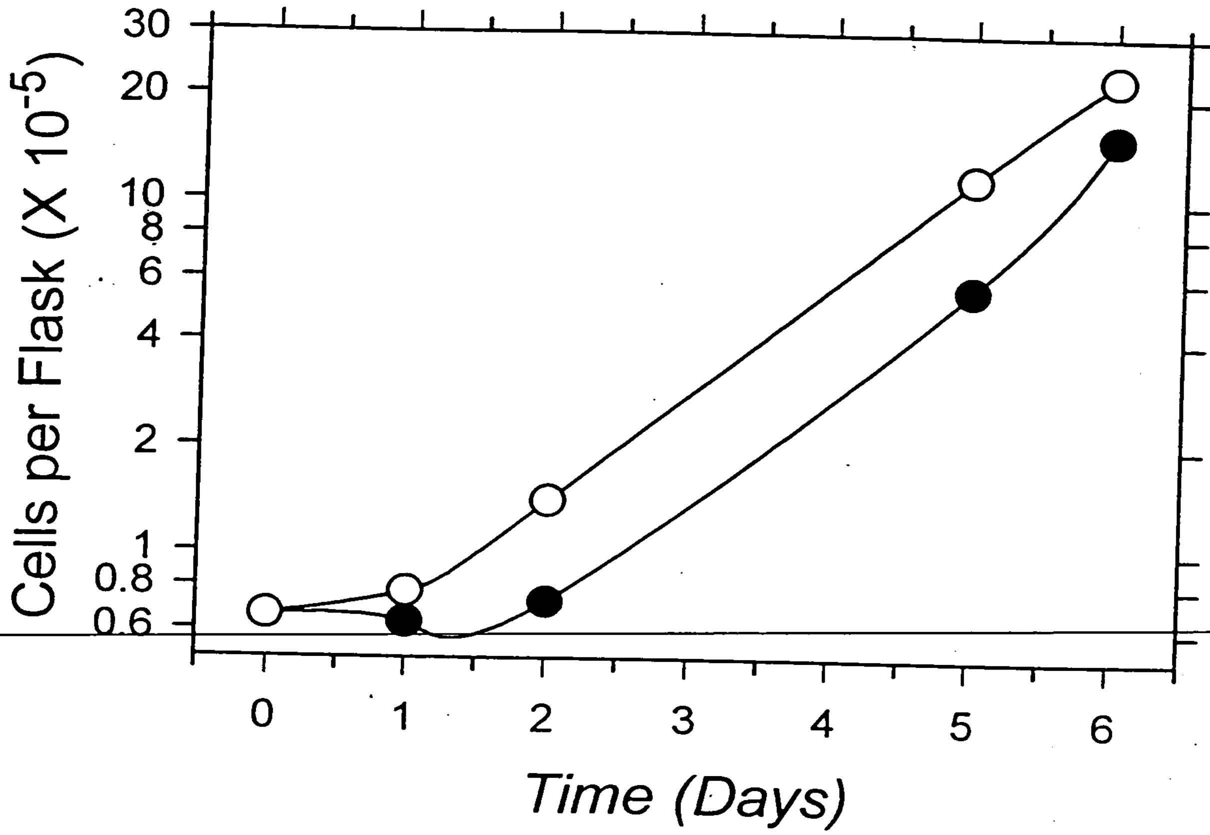

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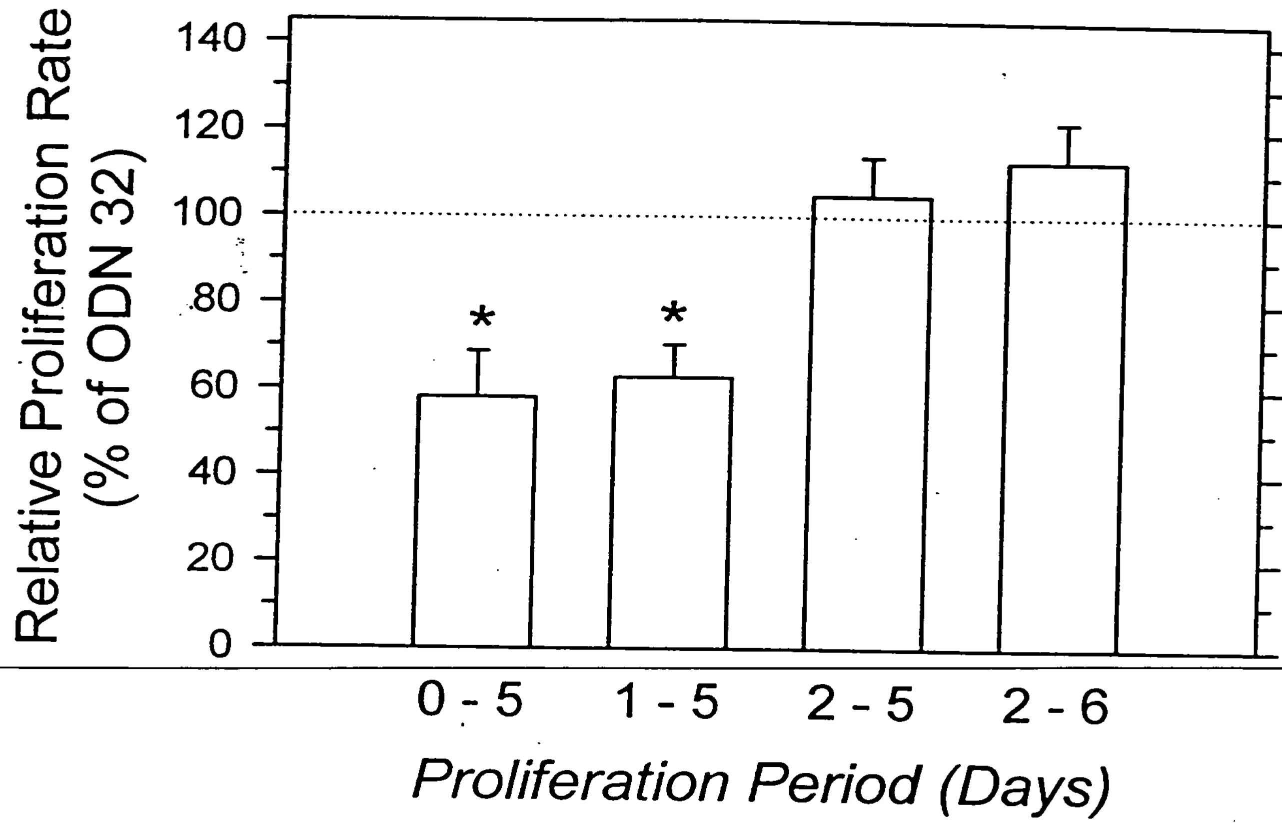

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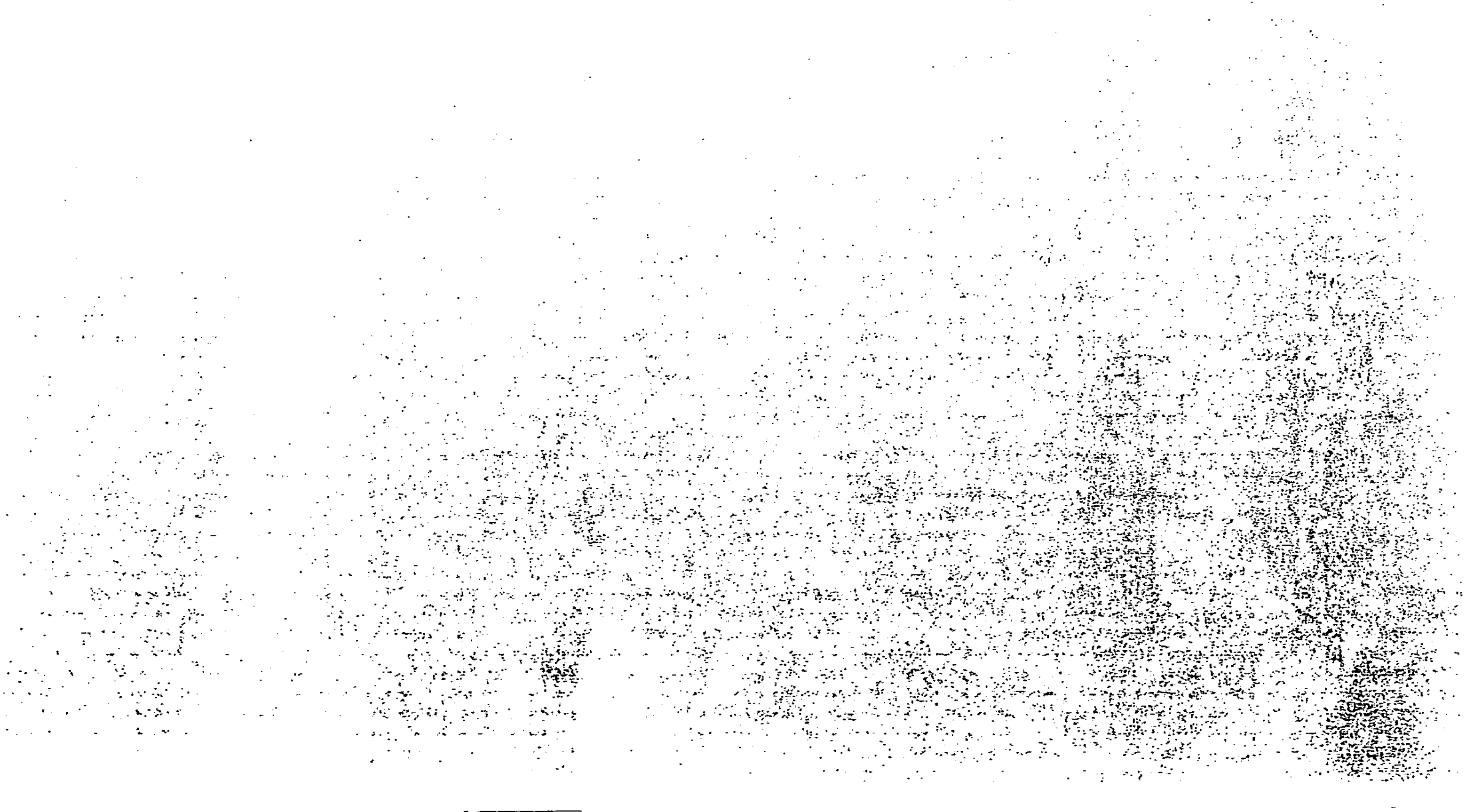

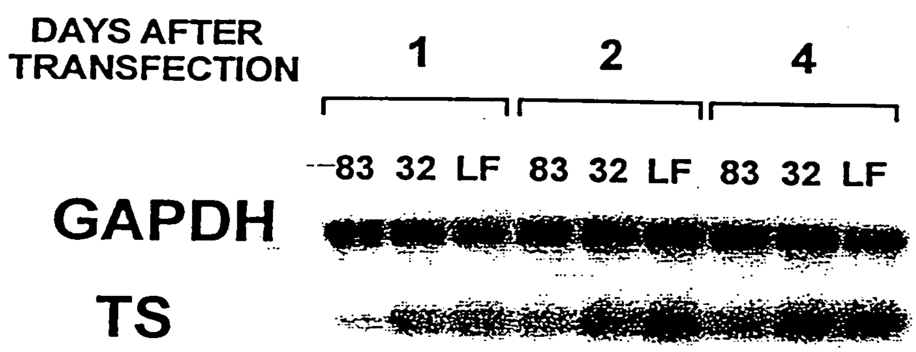

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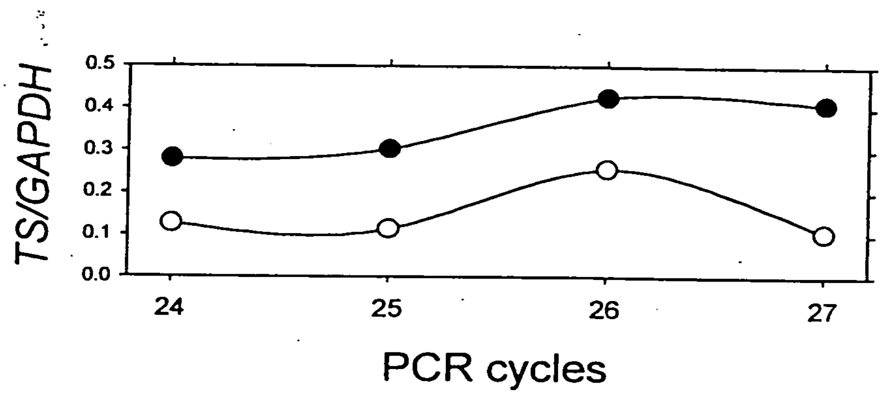

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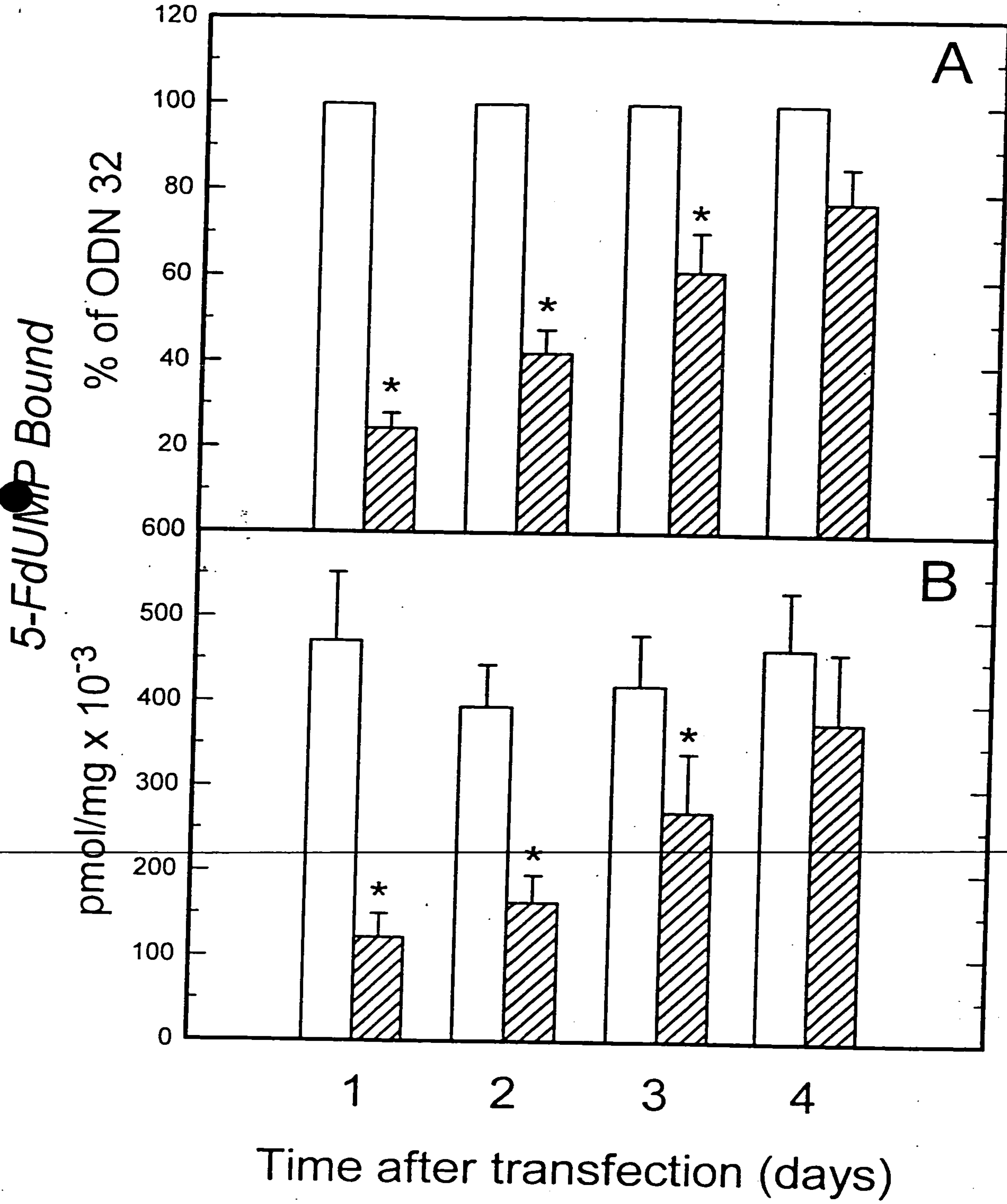
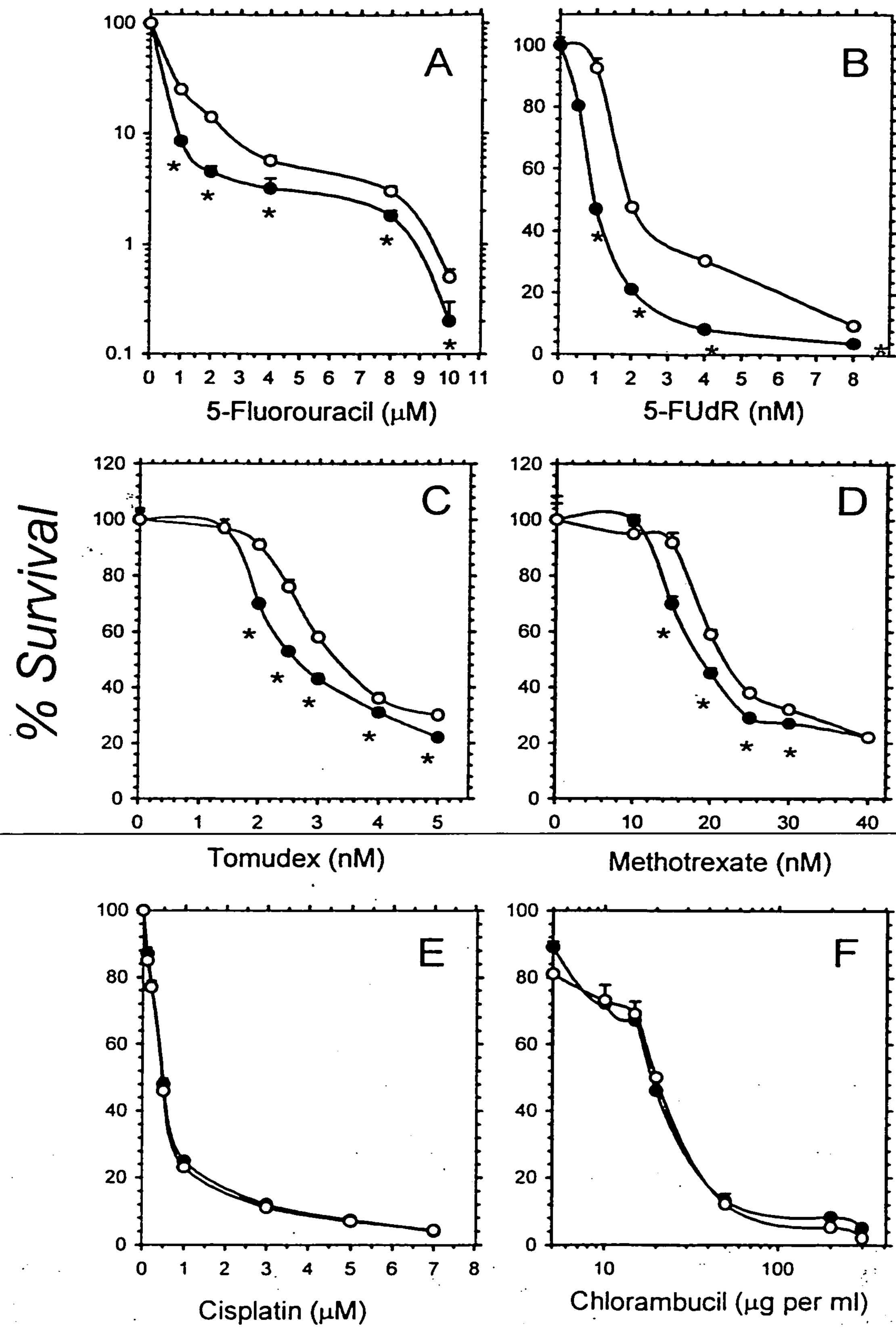

A
B
5-FdUMP Bound
% of ODN 32
pmol/mg x 10^{-3}
120
100
80
60
40
20
600
500
400
300
200
100
0
*
1
2
3
4
Time after transfection (days)


A
B
C
D
E
F
% Survival
5-Fluorouracil (μM)
5-FUdR (nM)
Tomudex (nM)
Methotrexate (nM)
Cisplatin (μM)
Chlorambucil (μg per ml)

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